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Clinical Chemistry 43:11
2021–2038 (1997)

Review

Molecular diagnostics of infectious diseases

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Over the past several years, the development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases. Microbial phenotypic characteristics, such as protein, bacteriophage, and chromatographic profiles, as well as biotyping and susceptibility testing, are used in most routine laboratories for identification and differentiation. Nucleic acid techniques, such as plasmid profiling, various methods for generating restriction fragment length polymorphisms, and the polymerase chain reaction (PCR), are making increasing inroads into clinical laboratories. PCR-based systems to detect the etiologic agents of disease directly from clinical samples, without the need for culture, have been useful in rapid detection of unculturable or fastidious microorganisms. Additionally, sequence analysis of amplified microbial DNA allows for identification and better characterization of the pathogen. Subspecies variation, identified by various techniques, has been shown to be important in the prognosis of certain diseases. Other important advances include the determination of viral load and the direct detection of genes or gene mutations responsible for drug resistance. Increased use of automation and user-friendly software makes these technologies more widely available. In all, the detection of infectious agents at the nucleic acid level represents a true synthesis of clinical chemistry and clinical microbiology techniques.

Over the past century microbiologists have searched for more rapid and efficient means of microbial identification. The identification and differentiation of microorganisms has principally relied on microbial morphology and growth variables. Advances in molecular biology over the past 10 years have opened new avenues for microbial identification and characterization *[1–5]*.

The traditional methods of microbial identification rely solely on the phenotypic characteristics of the organism. Bacterial fermentation, fungal conidiogenesis, parasitic morphology, and viral cytopathic effects are a few phenotypic characteristics commonly used. Some phenotypic characteristics are sensitive enough for strain characterization; these include isoenzyme profiles, antibiotic susceptibility profiles, and chromatographic analysis of cellular fatty acids *[6–13]*. However, most phenotypic variables commonly observed in the microbiology laboratory are not sensitive enough for strain differentiation. When methods for microbial genome analysis became available, a new frontier in microbial identification and characterization was opened.

Early DNA hybridization studies were used to demonstrate relatedness amongst bacteria. This understanding of nucleic acid hybridization chemistry made possible nucleic acid probe technology *[14–25]*. Advances in plasmid and bacteriophage recovery and analysis have made possible plasmid profiling and bacteriophage typing, respectively *[26–31]*. Both have proven to be powerful tools for the epidemiologist investigating the source and mode of transmission of infectious diseases *[26, 28, 30, 32–40]*. These technologies, however, like the determinations of phenotypic variables, are limited by microbial recovery and growth.

Nucleic acid amplification technology has opened new avenues of microbial detection and characterization *[1, 5, 41]*, such that growth is no longer required for microbial identification *[42–52]*. In this respect, molecular methods have surpassed traditional methods of detection for many fastidious organisms. The polymerase chain reaction (PCR) and other recently developed amplification techniques have simplified and accelerated the in vitro process of nucleic acid amplification. The amplified products, known as amplicons, may be characterized by various methods, including nucleic acid probe hybridization, analysis of fragments after restriction endonuclease digestion, or direct sequence analysis. Rapid techniques of nucleic acid amplification and characterization have significantly broadened the microbiologists' diagnostic arsenal.

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Received June 10, 1997; revision accepted July 18, 1997.

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whereas enzyme-labeled probed membrane may be visualized through either light or color production.

HYBRIDIZATION PROTECTION ASSAY (HPA)

HPA is a homogeneous format. The probe and the product are incubated together in a single test tube, and the binding of probe to the target is measured without further manipulation [150]. A probe labeled with an acridinium ester is added to a sample containing PCR products for identification. In a positive sample, the bound probe is protected from alkaline hydrolysis and, upon addition of peroxides, emits detectable light. The HPA does not require the binding of amplified DNA to a solid support by DNA capture or other means, can be performed in a few hours, and does not need to have excess unbound DNA probe removed [151, 152].

DNA ENZYME IMMUNOASSAY (DEIA)

DEIA is another newly developed system for detecting nucleic acid previously amplified by means of PCR [153]. An anti-dsDNA antibody exclusively recognizes the hybridization product resulting from the reaction between target DNA and a DNA probe. The final product is revealed by means of a colorimetric reaction [153]. The DEIA increases the sensitivity of a previous PCR by including enzymatic reactions. The hybridization between specific probe and PCR-amplified target DNA, as well as the formation of target DNA/probe hybrids and anti-dsDNA antibody complex, also enhances the specificity. The system is now manufactured by Sorin Biomedica Diagnostics in Europe and Incstar in the US. Variations on DEIA capture techniques have been explored recently [112, 144].

AUTOMATED DNA SEQUENCING TECHNOLOGY

Direct sequencing offers direct, rapid, and accurate analysis of amplification products. As described earlier, broad-range PCR amplifies conserved regions of a wide range of organisms [128, 133]. The amplicon sequence is first determined, then a DNA sequence-based phylogenetic analysis is performed and used to specifically identify the pathogen [154]. Current sequencing technologies include one of two approaches: electrophoretic separation, based on polyacrylamide slab gels or glass capillaries, and solid-phase sequencing, determined by matrix hybridization [128, 133].

SINGLE-STRAND CONFORMATIONAL POLYMORPHISMS (SSCP)

SSCP was first described by Orita et al. [155]. DNA is subjected to PCR with primers to a region of suspected polymorphism. The PCR products, which usually incorporate a detector marker, are examined after gel electrophoresis. Physical conformational changes in single-stranded DNA are based on the physiochemical properties of the nucleotide sequence. Variations in the physical conformation are reflected in differential gel migration. This technique is sensitive enough to detect single nucleotide substitutions. One area in which SSCP may prove to be of value is in the detection of mutations related to resistance mechanisms. SSCP, and variations on the technique, have been successfully used to examine the genes contributing to the multidrug resistance of *M. tuberculosis* [156, 157].

RFLP ANALYSIS

In postamplification RFLP analysis, the amplified DNA fragments are cut by a restriction endonuclease, separated by gel electrophoresis, and then transferred to a nitrocellulose or nylon membrane. The fragment(s) containing specific sequences may then be detected by using a labeled homologous oligonucleotide as a probe. Variations in the number and sizes of the fragments detected are referred to as RFLPs and reflect variations in both the number of loci that are homologous to the probe and the location of restriction sites within or flanking those loci [158]. An epidemiological application of RFLPs is discussed in more detail later.

Current Application of Molecular Diagnostics

CLINICAL MICROBIOLOGY

Traditionally, the clinical medical microbiology laboratory has functioned to identify the etiologic agents of infectious diseases through the direct examination and culture of clinical specimens. Direct examination is limited by the number of organisms present and by the ability of the laboratorian to successfully recognize the pathogen. Similarly, the culture of the etiologic agent depends on the ability of the microbe to propagate on artificial media and the laboratorian's choice of appropriate media for the culture. When a sample of limited volume is submitted, it is often not possible to culture for all pathogens. In such instances, close clinical correlation is essential for the judicious use of the specimen available.

Some microorganisms are either unculturable at present, extremely fastidious, or hazardous to laboratory personnel. In these instances, the diagnosis often depends on the serologic detection of a humoral response or culture in an expensive biosafety level II–IV facility. In community medical microbiology laboratories, these facilities may not be available, or it may not be economically feasible to maintain the special media required for culture of all of the rarely encountered pathogens. Thus, cultures are often sent to referral laboratories. During transit, fragile microbes may lose viability or become overgrown by contaminating organisms or competing normal flora.

The addition of molecular detection methods to the microbiology laboratory has resolved many of these problems. The exquisite sensitivity and specificity of many molecular methods allow the accurate detection of very small numbers of organisms. The direct detection of *M. tuberculosis* nucleic acid from the sputa of smear-negative patients with tuberculosis clearly illustrates this point [159–161]. The technology allows for the rapid and

Table 1. Commercially available and Mayo-developed amplification techniques for detection of microbial pathogens.

Organism detected	Manufacturer/ institute	Basic technique adopted	Trade mark/ name	Detection system	Contamination potential	Analytical sensitivity (lower detection limit) or testing range	Clinical sensitivity %	Clinical specificity %	Primary application	Additional comments/ information
HIV-1	Roche	PCR	Amplicor®	EIA	Low to moderate	<100 copies/mL[a]	>99[a]	No claims	Confirmatory testing	Customer service: 1-800-526-1247
	Roche	Quant. PCR	Monitor™	EIA	Low to moderate	400–750 000 copies/mL[b]	NA[c]	No claims	Quantitation during therapy	FDA-cleared
	Chiron	Quant. bDNA	Quantiplex™	EIA	Low	500–800 000 copies/mL[b]	NA[c]	95[b]	Quantitation during therapy	Customer service: 1-800-653-1353
	Organon Teknika	NASBA	HIV-1 QL	ECL	Moderate	80 copies/mL[b]	>99[b]	>99[b]	Confirmatory testing	Customer service: 1-800-682-2666
	Organon Teknika	Quant. NASBA	HIV-1 QT	ECL	Moderate	200-10^6 copies/mL[b]	NA[c]	>99[b]	Quantitation during therapy	
HCV	Roche	PCR	Amplicor®	EIA	Low to moderate	<200 copies/mL[a]	98%[a]	No claims	Confirmatory testing	
	Roche	Quant. PCR	Monitor™	EIA	Low to moderate	200-10^7 copies/mL[a]	NA[c]	No claims	Quantitation during therapy	
	Chiron	Quant. bDNA	Quantiplex™	EIA	Low	200 000–120 × 10^6 copies/mL[b]	NA[c]	98[b]	Quantitation during therapy	
	Mayo	RT-PCR		WB-ECL	Moderate	<20 copies/rxn[a]	>99[a]	>99[a]	Monitoring and confirmation	Contact: 1-507-284-1441
Mycobacterium tuberculosis	Roche	PCR	Amplicor®	EIA	Low to moderate	≥20 organisms/rxn[b]	88.9–100[b]	100[b]	Smear-positive and untreated patients only	FDA-cleared. Has been used on BACTEC broth culture
	Gen-Probe	TMA	MTD	HPA-ECL	Moderate	Unknown	95.5[b]	100[b]	Smear-positive and untreated patients only	FDA-cleared. Customer service: 1-800-523-5001
Chlamydia trachomatis	Roche	PCR	Amplicor®	EIA	Low to moderate	10–20 elementary bodies/rxn[b]	93.2[b]	98.4[b]	Monitoring and confirmation	FDA-cleared
	Abbott	LCR	LCX®	EIA	Moderate to high	Unknown	>95[a]	>99[a]	Monitoring and confirmation	FDA-cleared. Customer service: 1-800-527-1869
	Gen-Probe	TMA	AMP	HPA-ECL	Moderate	Unknown	86.7–99.2[b]	>99[b]	Monitoring and confirmation	Sensitivity varies in sexes and specimen source. FDA-cleared.
Neisseria gonorrhoeae	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Monitoring and confirmation	
	Abbott	LCR	LCX®	EIA	Moderate to high	Unknown	>95[a]	>99[a]	Monitoring and confirmation	FDA-cleared
Hepatitis B virus	Chiron	Quant. bDNA	Quantiplex™	EIA	Low	0.7–5000 × 10^6 copies/mL	NA[c]	98[b]	Quantitation and monitoring	
CMV	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Confirmatory testing	
	Roche	Quant. PCR	Monitor™	EIA	Low to moderate	No claims	NA[c]	No claims	Quantitation and monitoring	
	Mayo	PCR		WB-ECL	Moderate	<100 CMV genome copies/rxn	70–90[a]	>95[a]	Monitoring and confirmation	
HTLV-I/II	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Monitoring and confirmation	
Enterovirus	Roche	PCR	Amplicor®	EIA	Low to moderate	No claims	No claims	No claims	Monitoring and confirmation	
Borrelia burgdorferi	Mayo	PCR		WB-ECL	Moderate	<10 organisms	Variable[d]	>99[a]	Monitoring and confirmation	
HSV	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	70–90[a]	>99[a]	Monitoring and confirmation	
Bordetella pertussis	Mayo	PCR		WB-ECL	Moderate	<100 organisms/mL	>95[d]	95[a]	Monitoring and confirmation	

(continued)

Table 1. Continued

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Organism detected	Manufacturer/ institute	Basic technique adopted	Trade mark/ name	Detection system	Contamination potential	Analytical sensitivity (lower detection limit) or testing range	Clinical sensitivity %	Clinical specificity %	Primary application	Additional comments/ information
JC virus	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	75[a]	>95[a]	Monitoring and confirmation	
Babesia microti	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	>95[a]	>99[a]	Monitoring and confirmation	
HGE	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	>95[a]	>99[a]	Monitoring and confirmation	
Tropheryma whippelii	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	>95[a]	>99[a]	Monitoring and confirmation	More sensitive than histology for diagnosis
Epstein–Barr virus	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	Unknown[a]	>95[a]	Monitoring and confirmation	
Varicella-zoster virus	Mayo	PCR		WB-ECL	Moderate	<100 copies/mL	Unknown[a]	>95[a]	Monitoring and confirmation	

NASBA, nucleic acid sequence-based amplification; WB, Western blot; EIA, enzyme immunoassay; ECL, electrochemiluminescence; HTLV, human T cell lymphotropic virus; CMV, cytomegalovirus; HGE, human granulocytic ehrlichiosis; NA, not applicable; rxn, reaction.

[a] Based on Mayo's experience.
[b] Based on manufacturer's claim.
[c] For testing of seropositive patients only.
[d] 95% on synovial fluid specimens, 30% on cerebrospinal fluid and blood specimens from acute cases.
[e] Too few cases to evaluate sensitivity.

accurate identification of the etiologic agent in a time substantially shorter than traditional methods. This allows for earlier initiation of a focused antimicrobial regimen and decreases the likelihood of disease progression.

In selected situations, the limitations imposed by the ability of an organism to be cultured and the selection of appropriate media and culture conditions may be replaced by the use of molecular microbiology. Microbial DNA/RNA extracted from a clinical specimen may be analyzed for the presence of various organism-specific nucleic acid sequences regardless of the physiological requirements or viability of the organism *[136, 162–165]*. For example, the inability to culture and analyze the principal etiologic agent of non-A, non-B hepatitis limited medical advances in this area. Using various molecular methods, however, investigators have been able to isolate hepatitis C virus (HCV) nucleic acid *[166]*. Analysis and cloning of the HCV genome has provided the viral antigens necessary for the development of specific serologic tests *[167–169]*. Currently, RT-PCR allows for the identification, quantification, and sequence analysis of the HCV genome in infected individuals *[117, 170, 171]*.

Another unculturable microbe that has been specifically detected by PCR and probe analysis is *Tropheryma whippelii*, the causative agent of Whipple disease *[128, 172, 173]*. Because of the inability of this organism to grow on conventional media and the lack of a serologic test, diagnosis of Whipple disease is usually based on clinical and specific biopsy findings. Patients with Whipple disease often have gastrointestinal manifestations and undergo endoscopy. Small bowel biopsies reveal foamy histiocytes filling the lamina propria. The definitive diagnosis is made with the identification of non-acid-fast, periodic acid-shift-positive, diastase-resistant bacillary forms within the histiocytes. Extraintestinal Whipple disease, principally seen as arthritis and central nervous system involvement, may be missed entirely unless the clinician and pathologist have a high index of suspicion. Even so, the diagnosis in such instances may prove difficult. Advances in the molecular detection of *T. whippelii* have resolved this dilemma *[128, 172, 173]*. On the basis of bacterial 16S rRNA gene sequence analysis, an emerging pathogen, *Bordetella holmesii*, has been successfully identified in the immunocompromised hosts *[130, 131]*. Additionally, the DNA from a single clinical specimen, such as a knee fluid aspirate, may be tested for several etiologic agents in a differential diagnosis. In such instances, the specimen may also be analyzed for other fastidious and difficult-to-culture agents of infectious arthritis, such as *N. gonorrhea* or *Borrelia burgdorferi* *[14, 15, 60, 103, 125, 174]*.

As alluded to earlier, molecular methods may also be useful in instances of limited specimen volume *[175, 176]*. Even in low-volume specimens, enough DNA/RNA can often be extracted to allow performance of numerous molecular assays. However, though molecular methods are very sensitive, we emphasize that, like culture and direct